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Lipid Peroxidation in Adrenal and Testicular Microsomes

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Studies were carried out to determine the actions of and interactions between ascorbate, NADPH, Fe^{2+}, and Fe^{3+} on lipid peroxidation in adrenal and testicular microsomes. Ascorbate-induced malonaldehyde production was maximal in adrenal and testicular microsomes at an ascorbate concentration of $1 \times 10^{-4}M$. Fe^{2+}, at levels between 10^{-6} and $10^{-3}M$, produced concentration-dependent increases in lipid peroxidation in adrenal and testicular microsomes; Fe^{2+} had a far greater effect than Fe^{3+} in both tissues. In liver microsomes, by contrast, Fe^{2+} and Fe^{3+} had quantitatively similar effects on lipid peroxidation. NADPH alone had no effect on malonaldehyde production in adrenal or testicular microsomes. However, in the presence of low Fe^{2+} concentrations ($10^{-6}M$), NADPH stimulated adrenal malonaldehyde production. The stimulation of lipid peroxidation by NADPH plus low Fe^{2+} was not demonstrable in testicular microsomes nor in adrenal microsomes which had been heat-treated to inactivate microsomal enzymes. Testicular malonaldehyde production was stimulated by NADPH if Fe^{3+} (5×10^{-5} to $1 \times 10^{-3}M$) was added to the incubation medium; the stimulation was not demonstrable in heat-treated microsomes. Fe^{3+} plus NADPH had little effect on adrenal lipid peroxidation. In the presence of high Fe^{2+} levels ($10^{-3}M$), NADPH produced a concentration-dependent inhibition of adrenal lipid peroxidation; the inhibition was fully demonstrable in heat-treated microsomes. NADPH similarly inhibited ascorbate-induced lipid peroxidation in adrenal microsomes. In testicular microsomes, NADPH did not inhibit ascorbate or Fe^{2+}-induced lipid peroxidation. The results indicate that various endogenous substances may be important in the control of adrenal and testicular lipid peroxidation and that the nature of the regulation differs from tissue to tissue.

Introduction

The process of lipid peroxidation has been implicated in the hepatotoxicity of ethanol and carbon tetrachloride as well as in the toxic effects of many other chemicals (*1-3*). Lipid peroxidation has been shown to occur *in vitro* in a wide variety of tissues (*4-6*) but has been most extensively studied in the microsomal fraction of liver cells. Among the endogenous substances known to stimulate lipid peroxidation in hepatic microsomes is NADPH (*7*). The microsomal enzyme, NADPH-cytochrome c reductase, is required for NADPH enhancement of lipid peroxidation in liver microsomes. Therefore, inactivation of microsomal enzymes blocks the stimulatory effect of NADPH on lipid peroxidation. Other substances, including ascorbate and ferrous or ferric ion, also initiate hepatic microsomal lipid peroxidation, but non-enzymatically, and their effects, therefore, are unaltered by inactivation of microsomal enzymes.

Among the endocrine tissues, lipid peroxidation has been demonstrated in adrenocortical and testicular subcellular fractions (*5*). However, relatively little is known about the role of endogenous substances in controlling lipid peroxidation in steroid-producing tissues. Many of the enzymes involved in the production of steroid hormones are membrane-bound cytochrome P-450-containing mixed function oxidases. Since lipid peroxidation is a membrane-damaging process, factors affecting lipid peroxidation in the adrenal cortex or testis may have significant effects on steroidogenesis in those

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tissues. The following studies were carried out, therefore, to determine the actions of and interactions between ascorbate, NADPH, and ferrous or ferric ions on lipid peroxidation in adrenal and testicular microsomes. Effects in the adrenal and testes were compared with those in hepatic microsomes to determine if the factors affecting lipid peroxidation in the three tissues were similar.

Methods

Male English short-hair guinea pigs weighing approximately 1000 g were obtained from Camm Research Institute, Wayne, N.J. Animals were maintained under standardized conditions of light (6:00 am-6:00 pm) and temperature (22°C) on a diet of Wayne Guinea Pig Diet and water *ad libitum*. Guinea pigs were killed by decapitation between 8:00 and 9:00 am. Adrenals, liver, and testes were quickly removed and placed in cold 1.15% KCl-0.05*M* Tris-HCl buffer (pH 7.4) on ice. Tissues were trimmed free of connective tissue, weighed, and homogenized in KCl-tris buffer. Microsomes were obtained by differential centrifugation as previously described (*8*) and resuspended in 1.15% KCl-0.05*M* tris buffer (pH 7.4). In some experiments, microsomal enzymes were inactivated by heating the microsomal suspension at 70°C for 2 min. Enzyme inactivation was confirmed by the absence of detectable NADPH-cytochrome c reductase activity.

Microsomal suspensions were incubated in 25 ml Erlenmeyer flasks in a Dubnoff metabolic incubator at 37°C for 60 min under air. Total volume in each flask was 2.5 ml. Adrenal microsomes were incubated at a concentration of approximately 0.25 mg protein/ml, testicular microsomes at approximately 0.50 mg protein/ml, and liver microsomes at approximately 0.75 mg protein/ml. The protein concentrations employed were found to be optimal for lipid peroxidation in each tissue. As indicated, the following agents were added to the reaction flasks prior to incubation: $FeCl_3$ (Fisher Scientific Company, Fairlawn, N.J.), $FeSO_4$, *L*-ascorbic acid, and NADPH-type I (Sigma Chemical Company, St. Louis, Mo.). All of the effects of ferrous ion presented in this report were obtained using $FeSO_4$ as the source of Fe^{2+}. However, essentially identical results have also been obtained with $FeCl_2$.

Malonaldehyde production, as measured by the thiobarbituric acid test, served as an index of lipid peroxidation. Malonaldehyde was measured according to the method of Ottolenghi (*9*) as modified by Hunter et al. (*10*). Following incubation, 2.0 ml aliquots from each flask were transferred to centrifuge tubes containing 0.5 ml of 40% trichloroacetic acid and 0.25 ml of 5*N* HCl. After mixing, 0.5 ml of a 2% thiobarbituric acid solution was added and the samples were incubated for 20 min at 90°C. Following incubation the samples were cooled in an ice bath for 5 minutes and centrifuged at 30,000 *g* for 5 min in a Sorvall model SS-3 centrifuge. The amount of malonaldehyde in each sample was determined by measuring the optical density of the supernatants at 532 nm with a Gilford model 300-N spectrophotometer and by using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (*11*).

Results

Lipid peroxidation in both adrenal and testicular microsomes was stimulated by the addition of either ascorbate of ferrous (Fe^{2+}) ion to the incubation flasks (Table 1). The amount of lipid peroxidation was dependent upon the concentration of Fe^{2+} or ascorbate present in the reaction mixture. Malonaldehyde production, when expressed per ml of incubation medium, was similar in adrenal and testes at all concentrations of Fe^{2+} and ascorbate tested (Table 1). However, since the concentration of testicular microsomal protein incubated was twice that of adrenal microsomal protein, malonaldehyde production per mg of protein was greater in adrenal than testicular microsomes. Ascorbate stimulation was maximal in both tissues at a concentration of $1 \times 10^{-4}M$ and dropped off at higher or lower concentrations. Increasing concentrations of Fe^{2+} up to at least $1 \times 10^{-3}M$ caused increasing production of malonaldehyde. Solubility limitations in the reaction mixture prevented the use of higher concentrations of Fe^{2+}. Heat treatment of adrenal or testicular microsomes did not alter the stimulatory effects of Fe^{2+} or ascorbate (data not shown), indicating the nonenzymatic nature of the stimulation by each.

The time courses for Fe^{2+}- and ascorbate-induced lipid peroxidation were similar in adrenal and testicular microsomes. The time courses for adrenal lipid peroxidation are shown in Figure 1. Fe^{2+}-induced lipid peroxidation continued to increase for at least 90 min, while ascorbate-induced malonaldehyde production reached a maximum after about 30 min. We have established that neither adrenal nor testicular microsomes metabolize malonaldehyde under the incubation conditions described. Therefore, the time courses reflect only malonaldehyde production.

It has been reported that Fe^{2+} and Fe^{3+} are equipotent stimuli of lipid peroxidation in rat liver microsomes (*12*). The data in Table 2 indicate that Fe^{2+} and Fe^{3+} also have similar effects on lipid peroxidation in guinea pig liver microsomes. However, in both adrenal and testicular microsomes, Fe^{2+} was a much more potent stimulus for lipid

Table 1. Effects of varying concentrations of ascorbate, Fe^{2+}, or NADPH on malonaldehyde production by adrenal and testicular microsomes.[a]

	Malonaldehyde, nmole/ml						
	$1 \times 10^{-6}M$	$5 \times 10^{-6}M$	$1 \times 10^{-5}M$	$5 \times 10^{-5}M$	$1 \times 10^{-4}M$	$5 \times 10^{-4}M$	$1 \times 10^{-3}M$
Ascorbate							
Adrenal	1.1 ± 0.3	3.0 ± 0.2	4.0 ± 0.3	6.7 ± 0.2	7.4 ± 1.5	1.9 ± 0.1	0.4 ± 0.1
Testes	0.9 ± 0.1	1.7 ± 0.2	2.8 ± 0.6	4.0 ± 0.9	7.5 ± 2.0	6.5 ± 0.4	4.1 ± 0.4
Fe^{2+}							
Adrenal	0.8 ± 0.3	1.4 ± 0.1	1.7 ± 0.1	1.5 ± 0.2	2.8 ± 0.1	5.7 ± 0.7	12.1 ± 0.4
Testes	0.8 ± 0.1	1.8 ± 0.1	2.3 ± 0.2	3.0 ± 0.3	3.6 ± 0.6	7.8 ± 0.4	11.8 ± 0.5
NADPH							
Adrenal	ND[b]	ND	ND	ND	ND	0.1 ± 0.1	0.1 ± 0.1
Testes	ND	ND	ND	ND	ND	0.7 ± 0.5	0.7 ± 0.3

[a] Malonaldehyde was measured by the thiobarbituric acid test following a 60 min. aerobic incubation at 37°C. Values are the means ± SE of 4–6 determinations at various concentrations of ascorbate, Fe^{+2}, or NADPH.
[b] ND = not detectable.

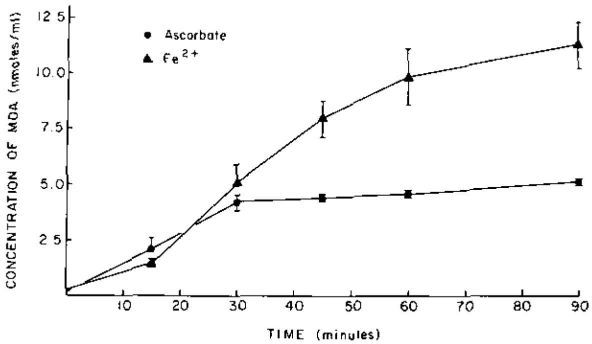

FIGURE 1. Time-courses for (Δ) ferrous ion ($10^{-3}M$) and (●) ascorbate ($10^{-4}M$) stimulation of malonaldehyde (MDA) production by adrenal microsomes (0.25 mg protein/ml) incubated aerobically at 37°C. Values are the means ± SE of 4-6 determinations.

peroxidation than Fe^{3+} (Table 2). Fe^{2+}-induced malonaldehyde production, when expressed per milligram of microsomal protein, in adrenal was approximately five times that in liver and twice as great in testes as in liver.

NADPH alone did not stimulate lipid peroxidation in adrenal or testicular microsomes (Table 1). However, when low levels (1×10^{-6}) of Fe^{2+} were added to the reaction mixture, NADPH produced concentration-dependent increases in lipid peroxidation in adrenal microsomes (Fig. 2). This stimulatory effect of NADPH in the presence of low levels of Fe^{2+} was not demonstrable in testicular microsomes, nor was it demonstrable in heat-treated adrenal microsomes (Fig. 2), suggesting an enzymatic process. Testicular malonaldehyde production was stimulated by NADPH if Fe^{3+} (5×10^{-5} to $1 \times 10^{-3}M$) was added to the incubation medium (Table 3). In adrenal microsomes, by contrast, Fe^{3+} (at concentrations from 1×10^{-6} to $1 \times 10^{-3}M$) only slightly enhanced NADPH-induced lipid peroxidation (data not shown). The interaction between Fe^{3+} and NADPH in testicular microsomes, like that between Fe^{2+} and NADPH in adrenal microsomes, was not demonstrable in heat-treated microsomes (Table 3 and Fig. 2, respectively), suggesting that enzymatic reactions were required.

Table 2. Effects of varying concentrations of Fe^{2+}, or Fe^{3+} on malonaldehyde production by liver, adrenal and testicular microsomes.[a]

	Malonaldehyde, nmole/ml			
	$1 \times 10^{-5}M$	$1 \times 10^{-4}M$	$5 \times 10^{-4}M$	$5 \times 10^{-3}M$
Liver				
Fe^{2+}	1.7 ± 0.2	1.5 ± 0.2	1.8 ± 0.2	6.8 ± 0.1
Fe^{3+}	1.6 ± 0.2	1.8 ± 0.2	2.2 ± 0.4	5.5 ± 0.3
Adrenal				
Fe^{2+}	1.8 ± 0.2	2.8 ± 0.2	5.9 ± 0.8	12.4 ± 0.5
Fe^{3+}	0.6 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.7 ± 0.2
Testes				
Fe^{2+}	2.3 ± 0.2	3.6 ± 0.6	7.8 ± 0.4	11.8 ± 0.5
Fe^{3+}	0.8 ± 0.1	0.9 ± 0.1	1.9 ± 0.1	1.8 ± 0.2

[a] Microsomes were incubated aerobically at 37°C for 60 min at optimal protein concentrations for lipid peroxidation (liver at approximately 0.75 mg/ml; adrenal at 0.25 mg/ml; testes at 0.50 mg/ml). Values are the means ± SE of 4-6 determinations.

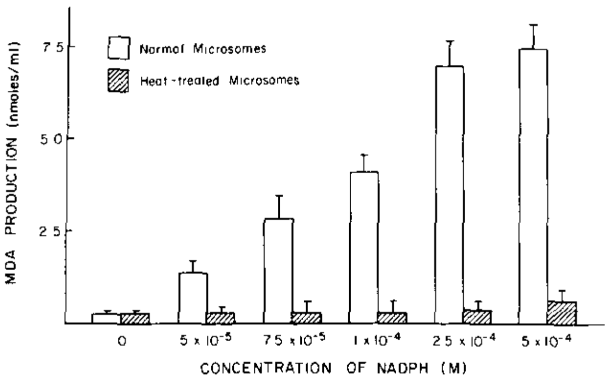

FIGURE 2. Concentration-dependent stimulation of malonaldehyde (MDA) production by NADPH in the presence of $10^{-6}M$ ferrous ion in normal or heat-treated adrenal microsomes (0.25 mg protein/ml). Malonaldehyde was determined following a 60 min aerobic incubation at 37°C. Values are the means ± SE of 4-6 determinations.

In contrast to the stimulatory effects of NADPH on lipid peroxidation in adrenal microsomes in the presence of low Fe^{2+} concentrations, in the presence of high levels ($1 \times 10^{-3}M$) of Fe^{2+}, NADPH inhibited lipid peroxidation (Fig. 3). This inhibition of Fe^{2+}-induced lipid peroxidation by NADPH was concentration-dependent and fully demonstrable in heat-treated microsomes (Fig. 3), indicating a nonenzymatic effect. NADPH similarly inhibited ascorbate ($1 \times 10^{-4}M$) - induced adrenal microsomal lipid peroxidation. In testicular microsomes, Fe^{2+} ($1 \times 10^{-3}M$)-induced lipid peroxidation was only slightly diminished by NADPH and ascorbate-induced malonaldehyde production in testicular microsomes was unaffected by any concentration of NADPH tested (data not shown).

Discussion

The results demonstrate that a number of endogenous substances previously found to affect lipid peroxidation in hepatic microsomes also influence lipid peroxidation in steroidogenic tissues, but that the specific effects vary from one tissue to another. An absolute dependence on non-heme iron for lipid peroxidation in hepatic microsomes was first suggested by Wills (*12*) and recently confirmed by Kornbrust and Mavis (*13*). In our studies, ferrous (Fe^{2+}) ion stimulated malonaldehyde production in guinea pig liver, adrenal, and testicular microsomes *in vitro*. The extent of lipid peroxidation was directly proportional to the Fe^{2+} concentration in all three tissues, but the amount of malonaldehyde produced varied with the source of the microsomes. Malonaldehyde production, when expressed per milligram of microsomal protein, was greatest in the adrenal, perhaps as a result of the high concentration of unsaturated fatty acids in the adrenal cortex (*14*). The extent of Fe^{2+}-induced testicular microsomal lipid peroxidation was greater than that in hepatic microsomes but less than adrenal lipid peroxidation.

In both adrenal and testicular microsomes, ascorbate-induced malonaldehyde production was maximal at an ascorbate concentration of $1 \times 10^{-4}M$. Higher concentrations resulted in a rapid decline in lipid peroxidation in the adrenal and a more gradual decline in testicular lipid peroxidation, probably as a result of the antioxidant properties of ascorbate being manifested. The normal level of ascorbate in the guinea pig adrenal is nearly $1 \times 10^{-2}M$, a concentration at which ascorbate may exert primarily antioxidant effects (*15,16*). However, upon stimulation by ACTH, the ascorbate concentration of the adrenal cortex decreases to levels which may promote lipid peroxidation (*17*). The normal level of ascorbate in the guinea pig testis is approximately $1 \times 10^{-3}M$ (*15*), a concentration which we have found to stimulate lipid peroxidation in testicular microsomes.

As previously reported for rat liver microsomes, Fe^{3+} and Fe^{2+} had similar effects on lipid perox-

Table 3. Effects of varying concentrations of NADPH on malonaldehyde production by normal or heat-treated testicular microsomes in the presence or absence of Fe^{3+} ($1 \times 10^{-}M$).[a]

	Malonaldehyde, nmole/ml					
	0	$1 \times 10^{-5}M$ NADPH	$5 \times 10^{-5}M$ NADPH	$1 \times 10^{-4}M$ NAPDH	$5 \times 10^{-4}M$ NADPH	$1 \times 10^{-3}M$ NADPH
Normal microsomes						
$-Fe^{3+}$	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4± 0.1	0.9 ± 0.1	0.9± 0.1
$+Fe^{3+}$	1.6 ± 0.2	1.6± 0.1	4.2 ± 0.2	6.9 ± 0.3	11.0 ± 0.7	12.7 ± 0.2
Heat-treated microsomes						
$+Fe^{3+}$	1.6 ± 0.1	1.5 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	1.8 ± 0.2	2.0 ± 0.1

[a] Malonaldehyde was determined following a 60-min aerobic incubation at 37°C. Values are the means ± SE of 4-6 determinations.

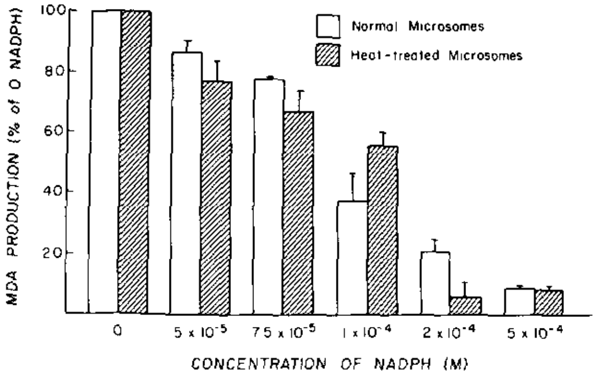

FIGURE 3. Effects of NADPH on ferrous ion ($10^{-3}M$) stimulation of malonaldehyde (MDA) production in normal or heat-treated adrenal microsomes (0.25 mg protein/ml). Malonaldehyde was determined following a 60 min aerobic incubation at 37°C. Values are the means ± SE of 4-6 determinations.

idation in guinea pig hepatic microsomes. However, Fe^{3+} had virtually no effect on lipid peroxidation in adrenal or testicular microsomes. It has been postulated that Fe^{3+} must be converted to Fe^{2+} to initiate lipid peroxidation (*18*). If that hypothesis is correct, the capacity for reducing Fe^{3+} must be much greater in hepatic than in adrenal or testicular microsomes. The reason for these differences is presently unknown.

The results of numerous studies have established that NADPH is a potent stimulus for lipid peroxidation in hepatic microsomes (*7*). Recently, Kornbrust and Mavis have proposed that the role of NADPH in lipid peroxidation is to maintain sufficient levels of iron in the reduced form to stimulate lipid peroxidation (*13*). Our observation that NADPH did not stimulate lipid peroxidation in adrenal or testicular microsomes unless exogenous iron was also present tends to support that hypothesis. However, with adrenal microsomes, NADPH produced far greater stimulation of lipid peroxidation in the presence of low (1×10^{-6} M) Fe^{2+} than Fe^{3+}, whereas with testicular microsomes the opposite pertained. The stimulatory effects of NADPH (in the presence of low Fe^{2+} or Fe^{3+}) on lipid peroxidation were not demonstrable in either adrenal or testes after heat treatment or microsomes, suggesting the involvement of enzymatic processes. Further studies will be necessary to determine the mechanisms responsible for the differential effects of Fe^{2+} and Fe^{3+} on NADPH-induced lipid peroxidation in adrenal and testicular microsomes.

In contrast to its stimulatory effects on lipid peroxidation in the presence of small amounts of iron, NADPH produced a concentration-dependent inhibition of adrenal malonaldehyde production when lipid peroxidation was initiated by high levels ($1 \times 10^{-3}M$) of Fe^{2+} or by ascorbate ($1 \times 10^{-4}M$). This inhibitory effect of NADPH, which was not seen in hepatic or testicular microsomes, was fully demonstrable in heat-inactivated adrenal microsomes, suggesting a non-enzymatic mechanism. We previously found that NADPH could inhibit the Fe^{2+}-induced oxidation of linoleic acid, indicating that NADPH may act as a direct antioxidant. Thus, NADPH appears to exert opposing actions on adrenal lipid peroxidation, the net effect depending on the level of iron present. It is not clear why the actions of NADPH on adrenal lipid peroxidation differ in the presence of high and low iron concentrations. However, since the stimulatory effect of NADPH requires microsomal enzyme activity, it is possible that high iron concentrations inhibit microsomal enzymes, shifting the balance in favor of the antioxidant effects of NADPH. Further studies are also necessary to determine why the inhibitory effects of NADPH on adrenal lipid peroxidation are not demonstrable in testicular microsomes under the same experimental conditions.

Although these studies indicate that interactions between a number of substances normally found in the adrenal cortex and testes can have substantial effects on lipid peroxidation in those tissues, the significance of lipid peroxidation in steroid-producing cells has yet to be established. Many of the enzymes required for steroidogenesis are membrane-bound mixed function oxidases, and the membrane destruction resulting from excessive lipid peroxidation is known to compromise the activities of such enzymes. It is possible, therefore, that lipid peroxidation could contribute to the control of steroid hormone synthesis and release. Further investigations are now needed into the regulation of lipid peroxidation in steroidogenic tissues and its relationship to hormone production and secretion.

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